

# PATENT SPECIFICATION

NO DRAWINGS

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## A method of manufacturing bacteriophages in solid dry form.

### COMPLETE SPECIFICATION

We, BIOGENA, narodni podnik, of No. 108, Tr. Wilhelma Piecka, Prague XII, Czechoslovakia, a Czechoslovakian Company, do hereby declare the invention, for which we  
5 pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

This invention relates to a method of  
10 manufacturing bacteriophages in solid dry form.

The object of the invention is to provide a method of manufacture of reliable and active preparations for the prevention and  
15 cure of infectious diseases which are caused by various kinds of pathogens (for instance all kinds of dysentery, typhus, paratyphus, and other Salmonellas, Vibrio cholerae, E. coli including the pathogen types thereof  
20 causing infectious diseases especially in children, Staphylococcus and Proteus). The fight against these microorganisms has been carried through hitherto by the application of chemotherapeutics and antibiotics which  
25 have been shown at the present time to be insufficiently active, especially in cases of chronic diseases and of microbe carriers. Phagotherapy has proved to be especially  
30 suitable for such purposes, and the preparations according to the invention fully solve all the related problems.

Therapeutic bacteriophages have been manufactured hitherto by static cultivation  
35 in a fluid medium; the form for administration is a fluid native Bacteriophage. Such a process of manufacture does not meet therapeutical requirements, for the following reasons:

- (1) difficulty in the manufacture of a  
40 large amount in a short time,
- (2) low values (titres) of bacteriophages and small activity in the organism,
- (3) a preparation in fluid form unsuitable for administration,
- 45 (4) easy reduction in value by contamina-

tion by strange microbial flora,

(5) the instability of the preparation and the limited duration of the activity, and

(6) difficult dosage of the preparation.

According to the present invention we  
50 provide a method of manufacturing a therapeutic bacteriophage preparation in solid dry form comprising cultivating a strain of pathogenic bacteria by submerged aerobic  
55 cultivation in an aqueous solution of a protein hydrolysate, effecting lysis of the bacteria by the corresponding bacteriophage, precipitating the bacteriophage from the lysate by means of an inorganic salt, and drying  
60 the precipitate.

Further according to the present invention we provide a method of manufacturing a therapeutic bacteriophage preparation in  
solid dry form comprising cultivating the bacteriophage by submerged aerobic culti-  
65 vation in an aqueous solution of a protein hydrolysate containing the corresponding bacteria, inactivating the bacteria of the culture by heating and by filtration, pre-  
70 cipitating the bacteriophage from the filtrate by means of an inorganic salt, and separating and drying the precipitate.

The method of manufacture according to the present invention enables substantial re-  
75 trenchments, from the economic point of view, of materials, time and labour. The preparations are highly active (the titres are substantially tripled) and stable. By the application of the preparations, the duration  
80 of the diseases and of the convalescence is reduced by approximately 75%. The preparations surpass the antibiotics and chemotherapeutics in their therapeutic effect, and they do not show the disadvantages of same.  
85 The method of manufacture according to the present invention meets, according to present epidemiological experiences, all the requirements for an effective phagotherapy of human epidemics.

The following is an example of treatment 90

in a dysentery epidemic of the *Shigella sonnei*:

Therapy by

	Bacteriophage	Chloramphenicol	Chemotherapy (using phthalyl- sulphathiazole)
5			
Number of treated persons -	27	24	16
Negative after 24 hours -	21	19	—
Negative after 48 hours -	6	—	—

10 finished by the bacteriophage

The submerged aerobic cultivation of the chosen bacterial strains is carried through in a fluid medium in special equipment in the cultivation tanks, which medium is 15 aerated (by means of oxygen, or by the mixture of oxygen with the air, or by air) and mixed up under strictly sterile conditions. The liquid medium for the cultivation of bacteria used for the production of bacteriophages is an aqueous solution of a protein hydrolysate, i.e. a hydrolysate of meat (beef), casein, soya beans, or the like, containing 200 mg% AN (amine-nitrogen). The subcultures of the bacterial strains in the 20 fluid medium, prepared in a suitable manner, are transferred to the cultivation tanks where the multiplication of the bacterial suspension runs through. The duration of this multiplication is specific for every bacterial species, otherwise the cultivation as such may be adapted to the technological conditions. The microbe concentration used for a submerged cultivation in a nutrient medium prior to the inoculation with bacteriophages, is 2.5 to 3 x 10<sup>9</sup>/ml; the amount of bacteriophages added to the suspension of the strain is 5 per cent of the content of the liquid cultivation medium. To the 25 multiplied bacterial suspension, the specific bacteriophage is added in an amount corresponding to its titre. The process of lysis which follows, runs through in similar conditions to the preceding multiplication process of the microbial mass. The process of phagolysis when using submerged cultivation is stopped at zero values of living microbe concentrations in the nutrient medium and when there is attained in the nutrient medium the same total nitrogen (TN) values 30 which were attained in it prior to the commencement of the cultivation. The end of the lysis is accompanied by the reduction of the number of microbes of the mass and by the clarification of the liquid medium which is, after inactivation of the microbes and 35 filtration, concentrated by means of a suitable inorganic salt, for instance, ammonium sulphate preferably at 0°C. The bacteriophage lysate is inactivated by moderately heating the bacteriophage suspension to 56°C in a water bath. It will be understood that the inactivation applies to the cells of the cultivated bacterial strain and the cells of bacteria disturbed by phagolysis, but not

to the bacteriophage itself which must remain viable in the cultivation liquid, without losing titre in vitro. The inorganic salt for the concentration of the liquid bacteriophage is used to form a saturated solution. The suitability of the salt is determined by its 65 ability to precipitate practically all bacteriophage particles of the cultivation medium. This concentrate is then dried *in vacuo* at room temperature. The dried mixture is put into suitable administration form, for instance, it is compressed into the tablets, and the tablets are coated with an enteric layer, or alternatively the dried mixture is filled into enteric capsules. To provide the enteric product, the tablets or capsules may be 70 coated with protective layers of graphite or cellphthal (acetylphthalylcellulose). In the case of parenteral application, the inorganic salt concentrate is desalted by dialysis (for local use), and freeze-dried. 75

The preparations according to the present invention are enteric tablets or capsules containing dried, purified monovalent bacteriophages effective against pathogenic bacteria. For each type of infectious disease, a monovalent preparation is made from the corresponding specific bacteriophages. The preparations according to the present invention are characterized by a high specificity and prompt activity in the organism; they do not show any undesirable clinical side-effects; they do not destroy the natural microorganisms of the gut and do not create resistant strains of the causative organisms of the disease being treated. These preparations keep their biological activity for a very long time. 85

The very easy and convenient manner of administration (per os), without the necessity of any medical supervision, and the safe dosage, are very advantageous for the patient. 90

The dried preparations of bacteriophages may be used any time for further cultivations. 95

#### EXAMPLE:

The Manufacture of a Dysenteric Phage Sonne. 100

A concentrated hydrolysate of meat (beef) produced enzymatically is diluted with water to a concentration of 200 mg% amino-nitrogen, and the quantity of AN after the dilution and the quantity of Cl are deter- 105 110

mined. Added to this solution is NaCl to a concentration of 0.5%, the pH is adjusted to 7.6 and a further 0.1%  $\text{Na}_2\text{HPO}_4$  is added. The solution is sterilized at 120° for 30 minutes, and forms a nutrient medium which is then used for cultivation as follows:

In the cultivation tank 8000 ml of the sterile nutrient medium (pH 7.6) is mixed up with 350-400 ml of a suspension of the dysenteric strain Sonne (Shigella sonnei) which has been multiplied statically in the fluid medium by a 5 hours incubation at 37°C. It is cultivated at this temperature while stirring and aerating. The control samples are taken off 90-120 minutes after the inoculation. The value of pH is determined or adjusted to 7.6. After 3 hours cultivation, when the number of microbes is about 2.5 milliards /1 ml, 400-500 ml of a specific dysenteric phage of Shigella sonnei are added, which phage has been prepared by a static or submerged aerobic process of the phagolysis of the strain Sonne in the aforesaid nutrient medium. The phagolysis is assessed at 2 hour intervals. The end of the phagolysis is indicated visually by the clarification of the medium and by the decrease of the number of microbes in 1 ml. Also, for verifying the course of phagolysis, the total nitrogen content (TN) in the cultivation medium is determined during the period of cultivation. The cultivation has been successfully completed when the TN values in the cultivation medium are approximately equal to said values in the cultivation medium prior to the beginning of cultivation. The culture of the bacteriophage is taken off from the tank in aseptic conditions, inactivated by mild heating in a water bath at 56°C, and filtered by means of Seitz filters. The assessment of the activity of the native bacteriophage is carried out according to the Czechoslovak standard rules. The native bacteriophage is concentrated and purified. The culture of the bacteriophage is cooled to 0°C and precipitated by solid ammonium sulphate until complete saturation, pH is adjusted by 4 M HCl to 6.9, and the whole left standing in the ice-box over night at 4°C.

The precipitate thus formed is centrifuged or filtered off at a temperature of 4°C. To the precipitate 10% of the wet weight of calcium gluconate is added and the mixture is dried *in vacuo* at room temperature. The dried mass is compressed with a filling material into the tablets which are coated by means of an enteric layer, or the dried mixture is filled into enteric capsules. The optimal amount of the phage contained in the tablet is determined by titration for each individual charge. The application dose may be regulated according to the amount of the phage in a tablet.

It is possible to cultivate and to treat analogously the specific bacteriophages of the other microbial kinds also, such as typhus, paratyphus and other Salmonellas, Vibrio cholerae, E. coli including the pathogen types thereof causing infectious diseases especially in children, Staphylococcus and Proteus.

#### WHAT WE CLAIM IS:

1. A method of manufacturing a therapeutic bacteriophage preparation in solid dry form comprising cultivating a strain of pathogenic bacteria by submerged aerobic cultivation in an aqueous solution of a protein hydrolysate, effecting lysis of the bacteria by the corresponding bacteriophage, precipitating the bacteriophage from the lysate by means of an inorganic salt, and drying the precipitate.

2. A method of manufacturing a therapeutic bacteriophage preparation in solid dry form comprising cultivating the bacteriophage by submerged aerobic cultivation in an aqueous solution of a protein hydrolysate containing the corresponding bacteria, inactivating the bacteria of the culture by heating and by filtration, precipitating the bacteriophage from the filtrate by means of an inorganic salt, and separating and drying the precipitate.

3. The method according to claim 2, wherein the precipitation is carried out while the filtrate is cool, preferably at a temperature of about 0°.

4. The method according to any preceding claim, wherein the inorganic salt used is ammonium sulphate.

5. The method according to any preceding claim, wherein the precipitated bacteriophage is mixed with 10 per cent of calcium gluconate and the mixture dried in vacuum at room temperature.

6. The method according to any preceding claim, wherein the product is formed as a tablet or capsule and is coated with a layer of substance resistant to gastric juice.

7. A therapeutic bacteriophage when prepared according to any preceding claim.

8. A method of manufacturing a therapeutic bacteriophage in solid dry form, according to the Example, or its obvious equivalents.

9. A therapeutic bacteriophage preparation in solid dry form obtained by the method of claim 8 or its obvious equivalents.

10. A solid, dry concentrate of therapeutic bacteriophage suitable for oral or parenteral administration in human and veterinary practice.

H. D. FITZPATRICK & CO.,  
Chartered Patent Agents,  
94, Hope Street, Glasgow, C.2.  
and

3 Gray's Inn Square, London, W.C.1.

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